(51) International Patent Classification 6 :	A1	(11) International Publication Number:	WO 98/31395
A61K 48/00, C12N 15/86		(43) International Publication Date:	23 July 1998 (23.07.98)

(21) International Application Number: PCT/EP98/00220

(22) International Filing Date: 16 January 1998 (16.01.98)

(30) Priority Data:
08/785,074   17 January 1997 (17.01.97)   US
60/042,247   31 March 1997 (31.03.97)   US

(71) Applicants *(for all designated States except US)*: RHONE–POULENC RORER S.A. [FR/FR]; 20, avenue Raymond Aron, F–92160 Antony (FR). INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE [FR/FR]; 101, rue de Tolbiac, F–75654 Paris Cedex 13 (FR).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: FINIELS, Françoise [FR/FR]; 7, rue Valentin Hauy, F–75015 Paris (FR). GIMENEZ–RIBOTTA, Minerva [FR/FR]; 474, avenue de la Justice de Castelnau, F–34000 Montpellier (FR). MALLET, Jacques [FR/FR]; 18, rue Charcot, F–75013 Paris (FR). PRIVAT, Alain [FR/FR]; 300, rue des Graves, F–34980 Saint Clément de Rivière (FR). REVAH, Frédéric [FR/FR]; 137, rue de Grenelle, F–75007 Paris (FR).

(74) Agent: SAVINA, Jacques; Rhône–Poulenc Rorer S.A., Direction Brevets, 20, avenue Raymond Aron, F–92165 Antony Cedex (FR).

(81) Designated States: AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, GH, GW, HU, ID, IL, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ADENOVIRAL–VECTOR–MEDIATED GENE TRANSFER INTO MEDULLARY MOTOR NEURONS

(57) Abstract

The present invention relates to methods and compositions for delivering nucleic acids to motor neurons by administering the nucleic acids to muscle tissue. The invention relates to methods for treating pathologies of the nervous system, such as trauma and neurodegenerative diseases.

Adenoviral-vector-mediated gene transfer into medullary motor neurons

Cross-Reference to Related Application

5   This application is a continuation-in-part of
08/785,074, filed 17 January 1997.

Field of the Invention

The present invention relates to methods of
10 delivering a nucleic acid to motor neurons comprising
administering the nucleic acid to muscle tissue. More
particularly, the invention relates to methods for
treating pathologies of the nervous system by
intramuscular administration of a therapeutic gene, and
15 gene transfer into medullary motor neurons. This
invention also relates to compositions comprising the
gene in a form suitable for intramuscular
administration.

20   ## Background of the Invention

Neurodegenerative Disease

Motor neuron diseases, such as amyotrophic lateral
sclerosis (ALS) and spinal muscular atrophy of infancy
25 (SMA), are often debilitating and resist curative
treatment. For example, ALS is heavily disabling and
invariably lethal. With an incidence of 2.5/100 000 in

constant increase, a prevalence of 6-10/100,000 (Leigh,

Pathogenic mechanisms in amyotrophic lateral sclerosis

and other motor neurons disorders in Neurodegenerative

diseases by CALNE, Saunders W.B. Eds, Philadelphia,

5   U.S.A., 1994), ALS affects 90,000 patients in developed

countries, mostly adults in their sixth decade. The

disease is characterized by a progressive motor neuron

degeneration leading to paralysis, to total loss of

motor and respiratory functions, and eventually to

10 death two to eight years after the appearance of the

first clinical signs (mean duration after onset three

years). ALS is of genetic origin in 5% of the patients,

and sporadic in 95% of the cases. Point mutations in

the gene encoding for Cu/Zn superoxide dismutase (SOD1)

15 localised on chromosome 21q22-1 are responsible for the

pathology in 20% of the familial cases (Rosen et al.,

Mutations in Cu/Zn superoxide dismutase gene are

associated with familial amyotrophic lateral sclerosis,

Nature, 362, 59-62, 1993, review in Rowland,

20 Amyotrophic lateral sclerosis: Human challenge for

neuroscience, Proc. Natl. Acad. Sci. USA, 92,

1251-1253, 1995). The pathophysiological basis of the

sporadic forms remains unknown. Although the use of

Rilutek provides ALS patients with a modest increase in

25 survival probability, there is no curative treatment

available for this disorder.

Spinal muscular atrophy of infancy is an autosomal

recessive disease, which in its most severe form (SMA
type 1) affects 1/16,000-25 000 infants in Europe and
North.America. SMA-1 patients manifest weakness before
three months of age and are never able to be maintained

5   in a sitting posture. Average life expectancy is 8
months, with 95% mortality before the second birthday
(review in Crawford and Pardo, The neurobiology of
childhood spinal muscular atrophy, Neurobiol. of
Disease, 3, 97-110, 1996). The disease is linked to

10   mutations in the SMN gene (Lefebvre et al.,
Identification and characterization of a spinal
muscular atrophy determining gene, Cell, 80, 155-165,
1995). There is no curative treatment available for
this disease.

15

Neurothrophic Factors

Neurotrophic factors have been suggested as
potential therapeutic agents for motor neuron diseases
(Thoenen et al., Exp. Neurology 124,47-55, 1993).

20   Indeed, embryonic motor neuron survival in culture is
enhanced by members of the neurotrophin family such as
brain derived neurotrophic factor (BDNF),
neurotrophin-3 (NT-3), NT-4 (NT-4/5), cytokines such as
ciliary neurotrophic factor (CNTF), leukaemia

25   inhibitory factor (LIF) and cardiotrophin-1, glial cell
line-derived neurotrophic factor (GDNF), insulin-like
growth factor-1 (IGF-1) and members of the FGF family

(review in Henderson, Neurotrophic factors as
therapeutic agents in amyotrophic lateral sclerosis:
potential and pitfalls. *In* Serratrice G.T. and Munsat
T.L. eds. *Pathogenesis and therapy of amyotrophic*
5   *lateral sclerosis.* Advances in Neurology, 68, pp.
235-240, 1995. Lippincott-Raven publishers,
Philadelphia; Pennica et al., Cardiotrophin-1, a
cytokine present in embryonic muscle, supports
long-term survival of spinal motoneurons. Neuron, 17,
10 63-74, 1996).

In vivo, a reduction of motoneuronal death
occurring naturally during embryonic development was
observed with CNTF (Oppenheim et al., Control of
embryonnic motoneuron survival in vivo by ciliary
15 neurotrophic factor.
Science, 251, 1616-1618, 1991), BDNF (Oppenheim et al.,
Brain-derived neurotrophic factor rescues developing
avian motoneurons from cell death. Nature, 360,
755-757, 1992), GDNF (Oppenheim et al., Developing
20 motor neurons rescued from programmed and
axotomy-induced cell death by GDNF. Nature, 373,
344-346, 1995), and cardiotrophin-1 (Pennica et al.,
1996). Protection from retrograde motor neuron death
after acute peripheral nerve axotomy in neonate rodents
25 was evidenced with several factors (Sendtner et al.,
Ciliary neurotroptuc factor prevents the degeneration
of motor neurons after axotomy, Nature 345, 440-441,

1990, Sendtner et al., Ciliary neurotrophic factor

prevents degeneration of motor neurons in mouse mutant

progressive motor neuronopathy. Nature, 358, 502-504,

1992; Sendtner et al., Brain-derived neurotrophic

5   prevents the death of motoneurons in newborn rats after

nerve section. Nature, 360, 757-759, 1992; Vejsada et

al., Quantitative comparison of the transient rescue

effects of neurotrophic factors on axotomised

motoneurons in vivo. Eur. J. Neurosci., 7, 108-115,

10   1995). Also, a protective effect of CNTF and/or BDNF

was described in two murine models of inherited

progressive motor degeneration (Sendtner et al., 1992;

Mitsumoto et al., Arrest of motor neuron disease in

wobbler mice cotreated with CNTF and BDNF. Science,

15   265, 1107-1110, 1994).

Data showing that neurotrophic factors enhance

motoneuronal survival under multiple experimental

conditions, suggest that these molecules could decrease

the vulnerability of motoneurons in human pathologies.

20   However the use of trophic factors in patients is

limited by their poor bioavailaility after systemic

administration.

Neurotrophic factors systemically administered

penetrate the nervous system with low yield because of

25   the presence of the blood-brain barrier. Only a very

small fraction of the injected factor reaches

motoneurons, most probably at the level of

neuromuscular synapses (Dittrich et al., Ciliary

neurotrophic cancer: pharacokinetics and acute phase

response. Ann. Neurol., 35, 151-163, 1994, Yan et al.,

1994). Furthermore trophic factors are rapidly degraded

5 and display a short half-life after systemic

administration (2.9 minutes for CNTF in rat Dittrich et

al., 1994, Cedarbaum et al., A double-blind

placebo-controlled clinical trial of subcutaneous

recombinant human ciliary neurotrophic factor (rHCNTF)

10 in amyotrophic lateral sclerosis. Neurology, 46, 1244-

1249, 1996). As a consequence high doses have to be

administered in order to have any possibility of

reaching therapeutic concentrations at the motoneuronal

levels. However, such doses are likely to produce

15 negative side effects. This has been illustrated in

clinical trials using recombinant CNTF injected to ALS

patients. The injected doses shown to produce a

therapeutic effect in animals (Mitsumoto et al, 1994)

were above toxicity threshold in human (Miller et al.,

20 A placebo-controlled trial of recombinant human ciliary

neurotrophic (rhCNTF) factor in amyotrophic lateral

sclerosis. Annals Neurol., 39, 256-260, 1996), and

adverse effects such as cough, asthenia, nausea, weight

loss and even increased death rate at the highest dose

25 were observed, while no beneficial effect of CNTF

treatment could be detected (Miller et al., 1996).

     The clinical use of neurotrophic factors thus

requires the development of suitable modes of *in vivo*
delivery. Therapeutic gene transfer offers potential
advantages over direct administration of the protein,
such as continuous and/or targeted production of the

5 desired transgene *in vivo*.

Gene Therapy

Gene therapy is rapidly emerging as an effective
approach for management and treatment of a variety of

10 diseases. Examples of effective gene therapy regimens
appear routinely in the literature (see for example
Roth et al., Nature Medicine, Vol. 2, 985-991 (1996)).

Gene therapy, including the administration of
modified viruses as vectors, constitutes a particularly

15 promising approach for treating neurodegenerative
diseases. Among the viruses in current use for gene
therapy are adenoviruses (Le Gal La Salle et al.,
Science 259, 988-990), herpes viruses, adeno-associated
viruses and retroviruses. Studies have shown that these

20 vectors, and in particular the adenoviruses, are
capable of infecting with a very high efficiency cells
of the central nervous system. These results have
enabled the development of methods for treating
pathologies of the central nervous system by direct

25 injection into the central nervous system (in
particular by stereotaxis) of recombinant adenoviruses
comprising a therapeutic gene (see WO94/08026, the

contents of which are incorporated herein by

reference).

    With respect to neurodegenerative diseases or

traumas associated with the spinal cord, gene therapy

5   provides a method to combat degeneration of the motor

neurons (motoneurons) by delivering therapeutic genes,

such as a gene encoding a neurotrophic factor or a

growth factor to motor neurons. However, prior methods

are limited by the lack of a simple method enabling

10 specific transfer of a gene into motor neurons. The

present invention overcomes this problem.

Summary of the Invention

15   The present invention describes a particularly

efficient method for the selective transfer of genes

into motor neurons.

    One aspect of the invention provides a method of

delivering a nucleic acid to mammalian motor neurons

20 comprising administering said nucleic acid to muscle

tissue, wherein said nucleic acid is delivered to said

motor neurons.

    Another aspect of the invention is a method of

delivering a nucleic acid to mammalian motor neurons

25 comprising administering said nucleic acid to muscle

tissue proximate to the site of a nerve linkage

associated with a chosen medullary functional level,

wherein said nucleic acid is delivered to said motor
neurons.

Another aspect of the invention is a method of
producing a protein in mammalian motor neurons

5   comprising administering a nucleic acid encoding said
protein to muscle tissue, wherein said nucleic acid is
delivered to said motor neurons and expressed. In a
preferred embodiment, the protein is produced at the
post synaptic ends of neuromuscular junctions.

10   The invention also provides a method of inducing
peripheral or collateral sprouting of motor axon
endings comprising administering a nucleic acid to
muscle tissue, wherein said nucleic acid induces
peripheral or collateral sprouting of motor axon

15   endings. In a preferred embodiment, the nucleic acid
encodes a protein, such as a neurotrophic factor or a
growth factor.

Another aspect of the invention is a method of
protecting against axonal degeneration comprising

20   administering a nucleic acid to mammalian muscle
tissue, wherein said nucleic acid is delivered to said
motor neurons and protects against axonal degeneration.

Still another aspect of the invention is a method
of treating an impairment of the nervous system

25   comprising administering a nucleic acid encoding a
neuroactive substance to mammalian muscle tissue,
wherein said nucleic acid is delivered to motor

neurons. Preferred embodiments are the treatment of

nerve damage and neurodegenerative diseases, such as

amyotrophic lateral sclerosis and spinal muscular

atrophy of infancy.

5   A preferred aspect of the invention is a method of

treating amyotrophic lateral sclerosis comprising

administering to muscle tissue of a mammal suffering

therefrom a replication defective adenovirus comprising

a gene encoding neurotrophin-3.

10

Brief Description of the Drawings

FIGURE 1: β-galactosidase labelling of longitudinal

15   sections (50 µm) of rat spinal cord at the lumbar

level, after intramuscular injection of adenovirus-β-

galactosidase into the gastrocnemial muscle,

- diffuse labelling of numerous motoneuronal cellular

bodies (Δ),

20   - more intense labelling of a few motor neurons at the

level of the cellular body and of the neurites,

revealing the typical morphology of the motor neurons

(Å).

25   FIGURE 2: identical to A, higher magnification.

β-galactosidase labelling of longitudinal sections (50

µm) of rat spinal cord at the lumbar level, after

intramuscular injection of adenovirus-β-galactosidase
into the gastrocnemial muscle.

FIGURE 3: β-galactosidase-immunocytochemical co-

5   labelling (Calcitonin Gene Related Peptide (CGRP) of
longitudinal sections (50 μm) of rat spinal cord at the
lumbar level, after intramuscular injection of
adenovirus-β-galactosidase into the gastrocnemial
muscle.

10

Figure 4: Survival curves of AD-NT3 treated *pmn* mice.

Figure 5: Amplitude of the muscle action potential
after supramaximal sciatic nerve stimulation in

15 adenoviral treated *pmn* animals.

Figure 6: Immune response following adenoviral
injection. β-galactosidase activity in muscle of
preinjected and non-preinjected mice.

20

Figure 7: Assesment of naked DNA injection.
        Panel A: Comparison of naked DNA to adenovirus.
        Panel B: Impact of batch preparation.
        Panel C: Comparison of CMV and RSV promoters in

25 new-born mice.
        Panel D: Comparison of CMV and RSV promoters in
adult mice.

<u>Figure 8:</u> Survival of *pmn*-mice after AdNT-3±AdCNTF
Intramuscular Injection

Suvival of *pmn*-mice in each treatment group is
5   expressed as a percentage of investigated animals
(100%). Survival of AdNT-3-treated animals (bold line;
n=16) and AdNT-3+AdCNTF cotreated animals (grey line;
n=11) is significantly different (p<0.005) from non-
treated (solid line; n=14) or AdlacZ vector injected
10   mice (dashed line; n=12). Triangles: mean time of
survival.

<u>Figure 9:</u> Number of myelinated fibers in phrenic nerves
after AdNT-3±AdCNTF treatment.
15   At day 25 the number of myelinated fibers in
phrenic nerves of non-treated and AdlacZ-injected *pmn*-
mice was much smaller (122, n=8 and 120, n=6,
respectively) than in non-affected littermates (263,
n=4). The nerves of treated *pmn*-mice, however,
20   contained more myelinated fibers than those of non-
treated *pmn*-mice (AdNT-3: 164, n=8, *, p<0.05; AdNT-
3+AdCNTF: 192, n=8, ***, p<0.001). Also at day 35, the
number of myelinated fibres was higher in AdNT-
3+AdCNTF-treated (157, n=10, p<0.01, **) than in non-
25   treated (118, n=10) or AdlacZ-injected *pmn*-mice (115,
n=8); this corresponds to a reduction in fiber loss by
20%. At this age the difference between *pmn*-mice that

had been treated with AdNT-3 alone (130, n=7) and all

control animals (n=18) was of borderline significance

(p=0.054). Small bars indicated SEM.

5   Figure 10: Electronmicrographs of phrenic nerves from

AdNT-3±AdCNTF-treated *pmn*-mice aged 25 days.

A: Non-treated *pmn*-mouse; B: AdlacZ-treated *pmn*-

mouse; C: Normal litter mate; D: AdNT-3-treated *pmn*-

mouse; E: AdNT-3+AdCNTF-treated *pmn*-mouse; F-G: High-

10 power views of nerves from non-treated (F) and AdNT-

3+AdCNTF-treated *pmn*-mice (G). Note increased number

of myelinated axons in treated (D,E,G) as compared to

non-treated (A,B,F) *pmn*-mice. The axons in treated

mice tended to be smaller than in normal and also in

15 non-treated *pmn*-mice; the cross-sectional area of the

nerve in non-treated *pmn*-mice was often larger than in

treated *pmn*-mice which reflected proliferation of

Schwann cells and formation of Büngner bands (A,F).

20 Figure 11: Terminal innervation pattern in muscles from

treated and non-treated *pmn*-mice.

Superficial gluteus muscles of pmn-mice (e-f) and

non-affected littermates (a,b) aged 4 weeks stained

with an acetylcholinesterase-silver method. a: Overview

25 of the endplate zone showing numerous enplates supplied

by long terminal axons. The sensory innervation of a

muscle spindle is also visible (left). b: Bundle of

five long axons emerging from a small nerve branch and
supplying 5 endplates. The axons of two endplates
closest to the nerve branch can only be followed for
short distances. c: Non-treated pmn-mouse. An
5   overview of the endplate zone shows loss of terminal
axons and isolated enplates. The nerve branch consists
of two axons only and contains debris of degenerated
axons. d: Non-treated pmn-mouse. Bundle of terminal
axons in a relatively well-preserved part of the
10 muscle. The innervation pattern resembles that in a
normal mouse (b), but the axons are irregularly shaped
and the terminal branches are rarified. e: AdNT3-
treated pmn-mouse. One terminal axon emerging from a
nerve branch (left) branches at two successive Ranvier
15 nodes and forms 3 and 4-5 branches, respectively
(arrows), and supplies a group of endplates. f:
AdNT3+AdCNTF-treated pmn-mouse. Three terminal axons
branch repeatedly and supply a large number of
endplates. The last Ranvier node of one of these axons
20 gives rise to three short branches which supply three
endplates, one of which by means of a terminal sprout
supplies a fourth endplate (arrow).
Calibration bar: 100μm (a,e) and 25μm (b, d-f).

25   Detailed Description of the Invention

The present invention describes a particularly

efficient method for the selective transfer of genes
into motor neurons. The invention demonstrates that it
is possible to specifically transfer a gene into the
motor neurons by administration into the muscle.

5   Applicants describe herein that adenoviruses are
advantageously absorbed at the level of the
neuromuscular junctions (motor endplates), and
transported up to the cellular bodies of the motor
neurons (ventral horn of the spinal cord) by retrograde

10 transport along the motoneuronal axons. Intramuscular
injections of recombinant adenoviruses expressing a
trophic factor provides a particularly attractive mode
of administration. After intramuscular injection
adenoviruses will infect myotubes, thus allowing the

15 trophic factor to be produced at the synaptic end of
motoneurons and to be released continuously in the
circulation. Intramuscular injections lead to
retrograde transport of injected recombinant
adenoviruses, allowing high yield infection of the

20 afferent motoneurons (Finiels et al., Specific and
efficient gene transfer strategy offers new
potentialities for the treatment of motor neurone
diseases. Neuroreport, 7, 373-378, 1995). This leads to
the production of the transgene within the spinal cord.

25   Intramuscular administration of a therapeutic gene
constitutes a new and very specific method for
infecting the motor neurons by retrograde transport.

The present invention enables one to target precisely
the medullary stage on which it is desired to act,
according to the location of the trauma and/or of the
degeneration. In particular, the present invention

5   advantageously enables one to specifically and
unilaterally infect the motor neurons of the different
medullary functional stages by following the precise
map of the neuromuscular junctions. The present
invention has been found to be less traumatic and more

10   specific than stereotaxic injection into the medullary
parenchyma, which is more diffuse and not restricted to
the motor neurons.

Definitions

15   The following defined terms are used throughout
the present specification, and should be helpful in
understanding the scope and practice of the present
invention.

     A "polypeptide" is a polymeric compound comprised

20   of covalently linked amino acid residues. Amino acids
have the following general structure:

$$
\begin{array}{c}
H \\
| \\
R-C-COOH \\
| \\
NH_2
\end{array}
$$

25
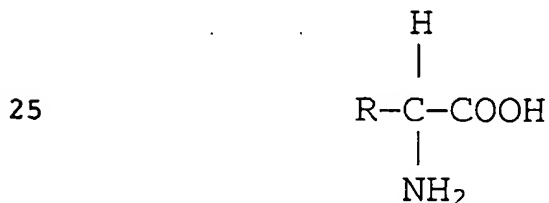
Amino acids are classified into seven groups on the
30   basis of the side chain R: (1) aliphatic side chains,

(2) side chains containing a hydroxylic (OH) group, (3) side chains containing sulfur atoms, (4) side chains containing an acidic or amide group, (5) side chains containing a basic group, (6) side chains containing an

5   aromatic ring, and (7) proline, an imino acid in which the side chain is fused to the amino group.

A "**protein**" is a polypeptide which plays a structural or functional role in a living cell.

The polypeptides and proteins of the invention may
10   be glycosylated or unglycosylated.

A "**variant**" of a polypeptide or protein is any analogue, fragment, derivative, or mutant which is derived from a polypeptide or protein and which retains at least one biological property of the polypeptide or

15   protein. Different variants of the polypeptide or protein may exist in nature. These variants may be allelic variations characterized by differences in the nucleotide sequences of the structural gene coding for the protein, or may involve differential splicing or

20   post-translational modification. The skilled artisan can produce variants having single or multiple amino acid substitutions, deletions, additions, or replacements. These variants may include, inter alia: (a) variants in which one or more amino acid residues

25   are substituted with conservative or non-conservative amino acids, (b) variants in which one or more amino acids are added to the polypeptide or protein, (c) variants in which one or more of the amino acids includes a substituent group, and (d) variants in which

30   the polypeptide or protein is fused with another polypeptide such as serum albumin. The techniques for obtaining these variants, including genetic (suppressions, deletions, mutations, etc.), chemical,

and enzymatic techniques, are known to persons having ordinary skill in the art.

If such allelic variations, analogues, fragments, derivatives, mutants, and modifications, including
5 alternative mRNA splicing forms and alternative post-translational modification forms result in derivatives of the polypeptide which retain any of the biological properties of the polypeptide, they are intended to be included within the scope of this invention.

10 A "nucleic acid" is a polymeric compound comprised of covalently linked subunits called nucleotides. Nucleic acid includes polyribonucleic acid (RNA) and polydeoxyribonucleic acid (DNA), both of which may be single-stranded or double-stranded. DNA includes cDNA,
15 genomic DNA, synthetic DNA, and semi-synthetic DNA. The sequence of nucleotides that encodes a protein is called the sense sequence.

"Regulatory region" means a nucleic acid sequence which regulates the expression of a second nucleic acid
20 sequence. A regulatory region may include sequences which are naturally responsible for expressing a particular nucleic acid (a homologous region) or may include sequences of a different origin (responsible for expressing different proteins or even synthetic
25 proteins). In particular, the sequences can be sequences of eukaryotic or viral genes or derived sequences which stimulate or repress transcription of a gene in a specific or non-specific manner and in an inducible or non-inducible manner. Regulatory regions
30 include origins of replication, RNA splice sites, enhancers, transcriptional termination sequences, signal sequences which direct the polypeptide into the secretory pathways of the target cell, and promoters.

A regulatory region from a "heterologous source"
is a regulatory region which is not naturally
associated with the expressed nucleic acid. Included
among the heterologous regulatory regions are
5   regulatory regions from a different species, regulatory
regions from a different gene, hybrid regulatory
sequences, and regulatory sequences which do not occur
in nature, but which are designed by one having
ordinary skill in the art.
10   A "vector" is any means for the transfer of a
nucleic acid according to the invention into a host
cell. The term "vector" includes both viral and
nonviral means for introducing the nucleic acid into a
cell *in vitro*, *ex vivo* or *in vivo*. Non-viral vectors
15 include plasmids, liposomes, electrically charged
lipids (cytofectins), DNA-protein complexes, and
biopolymers. Viral vectors include retrovirus, adeno-
associated virus, pox, baculovirus, vaccinia, herpes
simplex, Epstein-Barr and adenovirus vectors. In
20 addition to a nucleic acid according to the invention,
a vector may also contain one or more regulatory
regions, and/or selectable markers useful in selecting,
measuring, and monitoring nucleic acid transfer results
(transfer to which tissues, duration of expression,
25 etc.).

"Pharmaceutically acceptable carrier" includes
diluents and fillers which are pharmaceutically
acceptable for methods of administration, are sterile,
and may be aqueous or oleaginous suspensions formulated
30 using suitable dispersing or wetting agents and
suspending agents. The particular pharmaceutically
acceptable carrier and the ratio of active compound to

carrier are determined by the solubility and chemical

properties of the composition, the particular mode of

administration, and standard pharmaceutical practice.

A "**motor neuron**" is a neuron which controls the

5   movement of voluntary muscles.

General Molecular Biology

The techniques of recombinant DNA technology are

10 known to those of ordinary skill in the art. General

methods for the cloning and expression of recombinant

molecules are described in Maniatis (Molecular Cloning,

Cold Spring Harbor Laboratories, 1982), and in Ausubel

(Current Protocols in Molecular Biology, Wiley and

15 Sons, 1987), which are incorporated by reference.

Nucleic Acids

The present invention relates to the discovery

that instramuscular administration provides a means for

20 delivering a nucleic acid sequence to motor neurons.

The nucleic acid of the invention preferably encodes a

neuroactive substance; a substance capable of exerting

a beneficial effect on nerve cells. It may be a

substance capable of compensating for a deficiency in

25 or of reducing an excess of an endogenous substance.

Alternatively, it may be a substance conferring new

properties on the cells.

The neuroactive substance may be an antisense sequence or a protein. Among the proteins suitable for practice of the invention are growth factors, neurotrophic factors, cytokines, neurotransmitter

5   synthesizing enzymes, enzymes, neurotransmitter receptors and hormone receptors.

Preferably, the growth factor is a colony stimulating factor (G-CSF, GM-CSF, M-CSF, CSF, and the like), fibroblast growth factor (FGFa, FGFb) or

10   vascular cell growth factor (VEGF). Among the neurotrophic factors, the preferred factors are ciliary neurotrophic factor (CNTF), glial cell maturation factors (GMFa, b), GDNF, BDNF, NT-3, NT-5 and the like.

The neurotrophic factor NT-3 is particularly

15   preferred. The complete nucleotide sequence encoding NT-3 is disclosed in WO91/03569, the contents of which are incorporated herein by reference.

Preferred cytokines are the interleukins and interferons. Enzymes included within the scope of the

20   invention are the enzymes for the biosynthesis of neuro transmitters (tyrosine hydroxylase, acetylcholine transferase, glutamic acid decarboxylase) and the lysosomal enzymes (hexosaminidases, arylsulphatase, glucocerebrosidase, HGPRT). The enzymes involved in

25   the detoxification of free radicals (super oxide dismutase I, II or III, catalase, glutathione peroxidase) are preferred. Receptors include the

androgen receptors (involved in Kennedy's disease).

These proteins may be used in native form, or in the form of a variant or fragment thereof.

The neuroactive substance may also be an antisense

5   sequence.   The down regulation of gene expression using antisense nucleic acids can be achieved at the translational or transcriptional level. Antisense nucleic acids of the invention are preferably nucleic acid fragments capable of specifically hybridizing with

10   a nucleic acid encoding an endogenous neuroactive substance or the corresponding messenger RNA. These antisense nucleic acids can be synthetic oligonucleotides, optionally modified to improve their stability and selectivity. They can also be DNA

15   sequences whose expression in the cell produces RNA complementary to all or part of the mRNA encoding an endogenous neuroactive substance. Antisense nucleic acids can be prepared by expression of all or part of a nucleic acid encoding an endogenous neuroactive

20   substance, in the opposite orientation, as described in EP 140308. Any length of antisense sequence is suitable for practice of the invention so long as it is capable of down-regulating or blocking expression of the endogenous neuroactive substance. Preferably, the

25   antisense sequence is at least 20 nucleotides in length. The preparation and use of antisense nucleic acids, DNA encoding antisense RNAs and the use of oligo

and genetic antisense is disclosed in WO92/15680, the

contents of which are incorporated herein by reference.

     The nucleic acid may be of natural or artificial

origin. It may be especially genomic DNA (gDNA),

5   complementary DNA (cDNA), hybrid sequences or synthetic

or semisynthetic sequences. It may be of human, animal,

plant, bacterial or viral origin and the like. It may

be obtained by any technique known to persons skilled

in the art, and especially by screening libraries, by

10 chemical synthesis, or alternatively by mixed methods

including chemical or enzymatic modification of

sequences obtained by screening libraries. It is

preferably cDNA or gDNA.

15

Regulatory Regions

     Generally, the nucleic acids of the present

invention are linked to one or more regulatory regions.

Selection of the appropriate regulatory region or

20 regions is a routine matter, within the level of

ordinary skill in the art.

     The regulatory regions may comprise a promoter

region for functional transcription in the motor

neurons, as well as a region situated in 3' of the gene

25 of interest, and which specifies a signal for

termination of transcription and a polyadenylation

site. All these elements constitute an expression

cassette.

Promoters that may be used in the present
invention include both constituitive promoters and
regulated (inducible) promoters. The promoter may be
5   naturally responsible for the expression of the nucleic
acid. It may also be from a heterologous source. In
particular, it may be promoter sequences of eucaryotic
or viral genes. For example, it may be promoter
sequences derived from the genome of the cell which it
10   is desired to infect. Likewise, it may be promoter
sequences derived from the genome of a virus, including
the adenovirus used. In this regard, there may be
mentioned, for example, the promoters of the E1A, MLP,
CMV and RSV genes and the like.

15   In addition, the promoter may be modified by
addition of activating or regulatory sequences or
sequences allowing a tissue-specific or predominant
expression (enolase and GFAP promoters and the like).
Moreover, when the nucleic acid does not contain
20   promoter sequences, it may be inserted, such as into
the virus genome downstream of such a sequence.

Some promoters useful for practice of this
invention are ubiquitous promoters (e.g. HPRT,
vimentin, actin, tubulin), intermediate filament
25   promoters (e.g. desmin, neurofilaments, keratin, GFAP),
therapeutic gene promoters (e.g. MDR type, CFTR, factor
VIII), tissue-specific promoters (e.g. actin promoter

in smooth muscle cells), promoters which are
preferentially activated in dividing cells, promoters
which respond to a stimulus (e.g. steroid hormone
receptor, retinoic acid receptor), tetracycline-

5   regulated transcriptional modulators, cytomegalovirus
immediate-early, retroviral LTR, metallothionein, SV-
40, E1a, and MLP promoters. Tetracycline-regulated
transcriptional modulators and CMV promoters are
described in WO 96/01313, US 5,168,062 and 5,385,839,

10 the contents of which are incorporated herein by
reference.

Vectors

    As discussed above, a "vector" is any means for
15 the transfer of a nucleic acid according to the
invention into a host cell. Preferred vectors are
viral vectors, such as retroviruses, herpes viruses,
adenoviruses and adeno-associated viruses.

    Preferably, the viral vectors are replication
20 defective, that is, they are unable to replicate
autonomously in the target cell. In general, the genome
of the replication defective viral vectors which are
used within the scope of the present invention lack at
least one region which is necessary for the replication

25 of the virus in the infected cell. These regions can
either be eliminated (in whole or in part), be rendered
non-functional by any technique known to a person

skilled in the art. These techniques include the total

removal, substitution (by other sequences, in

particular by the inserted nucleic acid), partial

deletion or addition of one or more bases to an

5   essential (for replication) region. Such techniques

may be performed *in vitro* (on the isolated DNA) or *in

situ*, using the techniques of genetic manipulation or

by treatment with mutagenic agents.

    Preferably, the replication defective virus

10 retains the sequences of its genome which are necessary

for encapsidating the viral particles.

    The retroviruses are integrating viruses which

infect dividing cells. The retrovirus genome includes

two LTRs, an encapsidation sequence and three coding

15 regions (gag, pol and env). The construction of

recombinant retroviral vectors has been described: see,

in particular, EP 453242, EP178220, Bernstein et al.

Genet. Eng. 7 (1985) 235; McCormick, BioTechnology 3

(1985) 689, etc. In recombinant retroviral vectors,

20 the gag, pol and env genes are generally deleted, in

whole or in part, and replaced with a heterologous

nucleic acid sequence of interest. These vectors can

be constructed from different types of retrovirus, such

as, HIV, MoMuLV ("murine Moloney leukaemia virus" MSV

25 ("murine Moloney sarcoma virus"), HaSV ("Harvey sarcoma

virus"); SNV ("spleen necrosis virus"); RSV ("Rous

sarcoma virus") and Friend virus. Defective retroviral

vectors are disclosed in WO95/02697.

In general, in order to construct recombinant
retroviruses containing a nucleic acid sequence, a
plasmid is constructed which contains the LTRs, the
5   encapsidation sequence and the coding sequence. This
construct is used to transfect a packaging cell line,
which cell line is able to supply in trans the
retroviral functions which are deficient in the
plasmid. In general, the packaging cell lines are thus
10   able to express the gag, pol and env genes. Such
packaging cell lines have been described in the prior
art, in particular the cell line PA317 (US4,861,719);
the PsiCRIP cell line (WO90/02806) and the GP+envAm-12
cell line (WO89/07150). In addition, the recombinant
15   retroviral vectors can contain modifications within the
LTRs for suppressing transcriptional activity as well
as extensive encapsidation sequences which may include
a part of the gag gene (Bender et al., J. Virol. 61
(1987) 1639). Recombinant retroviral vectors are
20   purified by standard techniques known to those having
ordinary skill in the art.

The adeno-associated viruses (AAV) are DNA viruses
of relatively small size which can integrate, in a
stable and site-specific manner, into the genome of the
25   cells which they infect. They are able to infect a
wide spectrum of cells without inducing any effects on
cellular growth, morphology or differentiation, and

they do not appear to be involved in human pathologies.
The AAV genome has been cloned, sequenced and
characterized. It encompasses approximately 4700 bases
and contains an inverted terminal repeat (ITR) region
5 of approximately 145 bases at each end, which serves as
an origin of replication for the virus. The remainder
of the genome is divided into two essential regions
which carry the encapsidation functions: the left-hand
part of the genome, which contains the rep gene
10 involved in viral replication and expression of the
viral genes; and the right-hand part of the genome,
which contains the cap gene encoding the capsid
proteins of the virus.

The use of vectors derived from the AAVs for
15 transferring genes *in vitro* and *in vivo* has been
described (see WO 91/18088; WO 93/09239; US 4,797,368,
US 5,139,941, EP 488 528). These publications describe
various AAV-derived constructs in which the rep and/or
cap genes are deleted and replaced by a gene of
20 interest, and the use of these constructs for
transferring the said gene of interest in vitro (into
cultured cells) or in vivo, (directly into an
organism). The replication defective recombinant AAVs
according to the invention can be prepared by
25 cotransfecting a plasmid containing the nucleic acid
sequence of interest flanked by two AAV inverted
terminal repeat (ITR) regions, and a plasmid carrying

the AAV encapsidation genes (rep and cap genes), into a

cell line which is infected with a human helper virus

(for example an adenovirus). The AAV recombinants which

are produced are then purified by standard techniques.

5   The invention also relates, therefore, to an AAV-

derived recombinant virus whose genome encompasses a

sequence encoding a nucleic acid encoding a neuroactive

substance flanked by the AAV ITRs. The invention also

relates to a plasmid encompassing a sequence encoding a

10   nucleic acid encoding a neuroactive substance flanked

by two ITRs from an AAV. Such a plasmid can be used as

it is for transferring the nucleic acid sequence, with

the plasmid, where appropriate, being incorporated into

a liposomal vector (pseudo-virus).

15   In a preferred embodiment, the vector is an

adenovirus vector.

Adenoviruses are eukaryotic DNA viruses that can

be modified to efficiently deliver a nucleic acid of

the invention to a variety of cell types.

20   Various serotypes of adenovirus exist. Of these

serotypes, preference is given, within the scope of the

present invention, to using type 2 or type 5 human

adenoviruses (Ad 2 or Ad 5) or adenoviruses of animal

origin (see WO94/26914). Those adenoviruses of animal

25   origin which can be used within the scope of the

present invention include adenoviruses of canine,

bovine, murine (example: Mav1, Beard et al., Virology

75 (1990) 81), ovine, porcine, avian, and simian

(example: SAV) origin. Preferably, the adenovirus of

animal origin is a canine adenovirus, more preferably a

CAV2 adenovirus (e.g. Manhattan or A26/61 strain (ATCC

5   VR-800), for example).

Preferably, the replication defective adenoviral

vectors of the invention comprise the ITRs, an

encapsidation sequence and the nucleic acid of

interest. Still more preferably, at least the E1

10 region of the adenoviral vector is non-functional. The

deletion in the E1 region preferably extends from

nucleotides 455 to 3329 in the sequence of the Ad5

adenovirus (PvuII-BglII fragment) or 382 to 3446

(HinfII-Sau3A fragment). Other regions may also be

15 modified, in particular the E3 region (WO95/02697), the

E2 region (WO94/28938), the E4 region (WO94/28152,

WO94/12649 and WO95/02697), or in any of the late genes

L1-L5.

In a preferred embodiment, the adenoviral vector

20 has a deletion in the E1 region (Ad 1.0). Examples of

E1-deleted adenoviruses are disclosed in EP 185,573,

the contents of which are incorporated herein by

reference. In another preferred embodiment, the

adenoviral vector has a deletion in the E1 and E4

25 regions (Ad 3.0). Examples of E1/E4-deleted

adenoviruses are disclosed in WO95/02697 and

WO96/22378, the contents of which are incorporated

herein by reference. In still another preferred

embodiment, the adenoviral vector has a deletion in the

E1 region into which the E4 region and the nucleic acid

sequence are inserted (see FR94 13355, the contents of

5   which are incorporated herein by reference).

The replication defective recombinant adenoviruses

according to the invention can be prepared by any

technique known to the person skilled in the art

(Levrero et al., Gene 101 (1991) 195, EP 185 573;

10   Graham, EMBO J. 3 (1984) 2917). In particular, they

can be prepared by homologous recombination between an

adenovirus and a plasmid which carries, inter alia, the

DNA sequence of interest. The homologous recombination

is effected following cotransfection of the said

15   adenovirus and plasmid into an appropriate cell line.

The cell line which is employed should preferably (i)

be transformable by the said elements, and (ii) contain

the sequences which are able to complement the part of

the genome of the replication defective adenovirus,

20   preferably in integrated form in order to avoid the

risks of recombination. Examples of cell lines which

may be used are the human embryonic kidney cell line

293 (Graham et al., J. Gen. Virol. 36 (1977) 59) which

contains the left-hand portion of the genome of an Ad5

25   adenovirus (12%) integrated into its genome, and cell

lines which are able to complement the E1 and E4

functions, as described in applications WO94/26914 and

WO95/02697. Recombinant adenoviruses are recovered and purified using standard molecular biological techniques, which are well known to one of ordinary skill in the art.

Pharmaceutical Administration

The process according to the present invention enables one to target precisely the motor neurons of each medullary functional stage. Thus, according to the site of the impairment to be treated, the administration is made into a muscle carrying a nerve linkage with the said site. According to the present invention, it is now possible, by a judicious choice of various injections, to infect specifically and unilaterally a large number of medullary motor neurons distributed over the various levels.

In a preferred embodiment, administration into the muscles of the upper limbs (biceps, triceps) makes it possible to transfer a gene into the motor neurons at the cervical level; administration into the muscles of the thorax (pectoral muscles) makes it possible to transfer a gene into the motor neurons at the thoracic level; or administration into the muscles of the lower limbs (gastrocnemial muscles) makes it possible to transfer a gene into the motor neurons at the lumbar and sacral levels.

Other muscles may of course be used for

administration into these motor neurons, and other

motor neurons may also be targeted. To this end, it is

possible to use precise maps of the neuromuscular

junctions in order to determine, depending on the

5   medullar stage targeted, the most appropriate muscle(s)

for the administration. Such maps are accessible to

persons skilled in the art (see especially

Nicholopoulos et al., J. Comp. Neurol. 217, 78-85;

Peyronnard et Charon, Exp. Brain Res. 50, 125-132).

10   Depending on the medullar stage which it will prove

convenient to infect, one or more muscles known to be

innervated by the stage in question can thus be chosen.

The intramuscular administration can be carried

out in various ways. According to a first embodiment,

15   it is performed by injection at several points of the

same muscle so as to affect a very large number of

motor endplates. This embodiment is particularly

efficient when the point of insertion of the nerve into

the muscle considered is not identifiable. When the

20   point of insertion of the nerve can be located, the

administration is advantageously carried out by one or

more injections at or near the said point. According to

this embodiment, the efficiency of the transfer is

greater because a high proportion of vector

25   administered is absorbed at the level of the

neuromuscular junction.

In a preferred embodiment of the present

invention, the intramuscular administration is carried
out by injections at several points of the same muscle.

In another preferred embodiment of the present
invention, the intramuscular administration is carried
5 out by injection(s) at or near the point of insertion
of the nerve.

A preferred subject of the present invention is a
method for the transfer of nucleic acids into motor
neurons comprising the muscular administration of an
10 adenoviral vector incorporating the said nucleic acid
into its genome. Preferably, the method according to
the invention is carried out by injection(s) at several
points of the same muscle, or when the point of
insertion of the nerve can be located, by one or more
15 injections at the level of or close to the said point.

Pharmaceutical Compositions

For their use according to the present invention,
the nucleic acids, either in the form of a vector or
20 naked DNA, are preferably combined with one or more
pharmaceutically acceptable carriers for an injectable
formulation. These may be in particular isotonic,
sterile, saline solutions (monosodium or disodium
phosphate, sodium, potassium, calcium or magnesium
25 chloride and the like or mixtures of such salts), or
dry, especially freeze-dried compositions which upon
addition, depending on the case, of sterilized water or

physiological saline, allow the constitution of

injectable solutions.

The preferred sterile injectable preparations can

be a solution or suspension in a nontoxic parenterally

5   acceptable solvent or diluent. Examples of

pharmaceutically acceptable carriers are saline,

buffered saline, isotonic saline (e.g. monosodium or

disodium phosphate, sodium, potassium, calcium or

magnesium chloride, or mixtures of such salts),

10   Ringer's solution, dextrose, water, sterile water,

glycerol, ethanol, and combinations thereof. 1,3-

butanediol and sterile fixed oils are conveniently

employed as solvents or suspending media. Any bland

fixed oil can be employed including synthetic mono- or

15   di-glycerides. Fatty acids such as oleic acid also

find use in the preparation of injectables.

The virus doses used for the administration may be

adapted as a function of various parameters, and in

particular as a function of the site (muscle) of

20   administration considered, the number of injections,

the gene to be expressed or alternatively the desired

duration of treatment. In general, the recombinant

adenoviruses according to the invention are formulated

and administered in the form of doses of between 10^4 and

25   10^{14} pfu, and preferably 10^6 to 10^{11} pfu. The term pfu

(plaque forming unit) corresponds to the infectivity of

a virus solution, and is determined by infecting an

appropriate cell culture and measuring, generally after

15 days, the number of plaques of infected cells. The

technique for determining the pfu titre of a viral

solution are well documented in the literature.

5   In a preferred embodiment, the composition

comprises an adenovirus comprising the NT-3 gene (AdNT-

3) in a concentration of about 1×10^9 pfu/100µl.

The nucleic acid can also be administered as a

naked DNA. Methods for formulating and administering

10 naked DNA to mammalian muscle tissue are disclosed in

US patents 5,580,859 and 5,589,466, the contents of

which are incorporated herein by reference.

The compositions according to the invention are

particularly useful for administration to motor neurons

15 as described above.

Treatment of Motor Neuron Impairments

The process according to the present invention is

particularly advantageous for the treatment of

20 medullary traumas or of motoneuronal degeneration

diseases. Medullary traumas correspond more

particularly to sections at the level of the motor

neurons which deprive them of their afferences coming

from the higher centres and cause their degeneration.

25 The transfer of genes encoding growth factors, for

example, into the sublesional motor neurons by

retrograde transport according to the invention now

offers the possibility of reducing or even preventing

this degeneration.

Neuropathies of the motor neuron include

amyotrophic lateral sclerosis, spinal amyotrophias type

5 I (Werdnig Hoffman disease), type II or III (Kugelberg-

Welander disease), bulbar spinal amyotrophias (such as

Kennedy's disease). The transfer of genes encoding

growth factors or other molecules known to exert a

neurotrophic effect on the motor neuron undergoing

10 degeneration according to the present invention also

offers a new route for the treatment of this type of

pathology.

The efficacy of the process of the invention can

be demonstrated on animal models, such as a model of

15 partial or complete section of the spinal cord,

(Wobbler mouse - animal model for studying amyotrophic

lateral sclerosis (Leestma J.E., Am. J. Pathol., 100,

821-824)); the mnd mouse (motor neuron degeneration:

animal model for studying amyotrophic lateral sclerosis

20 (Messer et al., 1992, Genomics. 18, 797-802)); pmn

mouse (progressive motor neuron neuropathy: animal

model for studying motor neural degeneration during

development), and SOD* mice: transgenic mice expressing

mutated forms of Cu/Zn SOD responsible for familial

25 forms of amyotrophic lateral sclerosis, as illustrated

in the examples. The incorporation, tolerance and

safety for man can be tested on *in vitro* models of

culture of human embryonic medullary neurons.

Examples

The present invention will be described in greater

5   detail with the aid of the following examples which

should be considered as illustrative and nonlimiting.

Example 1 demonstrates infection and retrograde

transport within motor neurons following intramuscular

injection using a replication defective adenovirus

10   comprising a marker gene. Example 1 further

demonstrates the effect of adenovirus vectors on host

immune system and inflammatory responses. Example 2

demonstrates the efficacy of an AdNT-3 in two animal

models of amyotrophic lateral sclerosis. Example 3

15   demonstrates marker gene expression following

administration of naked DNA to muscle tissue.

Example 1. Injection of adenovirus - β-galactosidase

20   into the gastrocnemial muscle in intact rats or in rats

having undergone a thoracic hemisection of the spinal

cord.

This example describes the transfer of the

β-gal gene at the level of the lumbar motor neurons by

25   administration of an adenovirus incorporating the said

gene into the gastrocnemial muscle.

More particularly, the study was carried out

39

on a model of partial or complete section of rat spinal

cord performed at the low thoracic level which has the

effect of paralysing the animal in one or both of its

lower limbs. Such a section deprives the motor neurons

5   of their afferences coming from the upper centres and

bring about their degeneration. The administration was

carried out so as to infect the sublesional motor

neurons by retrograde transport.

The adenoviral vector used in this example is

10   the Ad.RSV.βgal vector. This vector lacks sequences

necessary for its replication, but comprises the

sequences necessary for penetrating into the cells

infectable by the said vector as well as all the

essential sequences necessary for the encapsidation of

15   this adenovirus. It also carries, under the control of

the RSV promoter, the E. coli β-galactosidase gene. The

construction of the defective recombinant adenovirus

Ad.RSVβgal has been described in the literature

(Stratford-Perricaudet et al., J. Clin. Invest. 90

20   (1992) 626). Briefly, the adenovirus Ad.RSVβgal is a

defective recombinant adenovirus (from which the E1 and

E3 regions have been deleted) obtained by homologous

recombination in vivo between the mutant adenovirus Ad-

dl324 (Thimmappaya et al., Cell 31 (1982) 543) and the

25   plasmid pAd.RSVβgal (Akli et al., 1993).

The plasmid pAd.RSVβgal contains, in the 5' → 3'

orientation,

       - the PvuII fragment corresponding to the
left end of the Ad5 adenovirus comprising: the ITR
sequence, the origin of replication, the encapsidation
signals and the enhancer E1A;

5   - the gene encoding β-galactosidase under the
control of the RSV promoter (Rous sarcoma virus),

       - a second fragment of the Ad5 adenovirus
genome which allows homologous recombination between
the plasmid pAd.RSVβgal and the adenovirus dl324.

10   After linearization with the ClaI enzyme, the
plasmid pAd.RSVβgal and the adenovirus dl324 are co-
transfected into the line 293 in the presence of
calcium phosphate in order to allow homologous
recombination. The recombinant adenoviruses thus

15 generated are selected by plaque purification. After
isolation, the recombinant adenovirus DNA is amplified
in the cell line 293, which leads to a culture
supernatant containing the unpurified recombinant
defective adenovirus having a titre of about

20 10^{10} pfu/ml. The viral particles are then purified by
centrifugation on a caesium chloride gradient according
to known techniques (see especially Graham et al.,
Virology 52 (1973) 456). The adenovirus was then used
in purified form in a phosphate buffered saline (PBS).

25   Three injections of adenovirus Ad-RSV-β-gal ($10^{?}$
pfu per injection) were performed into the
gastrocnemial muscle, just after the animal has (or

otherwise) undergone a hemisection of the spinal cord
(low thoracic level, which has the effect of paralysing
the animal in one of its lower limbs). 9 µl of
adenovirus are injected per point of injection with a
5   Hamilton syringe.

The animals were sacrificed (perfusion 4%
paraformaldehyde) four days after injection, minimum
time for the retrograde transport to occur from the
muscle to the spinal cord. Three blocks of spinal cord
10 were cut longitudinally at the cervical, thoracic and
lumbar levels, into sections 50 µm thick. The sections
were treated for revealing the β-galactosidase which
makes it possible to visualize the cells which have
been infected by the virus. Some sections were
15 furthermore subjected to an anti-Calcitonin Gene
Related Peptide (CGRP) immunocytochemistry which makes
it possible to specifically label the motor neurons.

The β-galactosidase was revealed using its
substrate, X-gal, and the product of the reaction gives
20 a blue colour.

The Calcitonin Gene Related Peptide, CGRP, is a
neurotransmitter, a specific marker for the motor
neurons. It is revealed by immunocytochemistry with a
secondary antibody coupled to peroxidase and as enzyme
25 substrate diaminobenzidine; the product of the reaction
gives a chestnut colour.

The revealing of β-galactosidase made it possible

to visualize the presence of the infected motor

neurons, exclusively at the sublesional lumbar level in

the case of the hemisectioned rats, and on the side

corresponding to the injection.

5   Two types of labelling were obtained, a diffuse

labelling of the cellular body of a large number of

motor neurons, and a more intense labelling of the

cellular body and of the neurites of a more limited

number of motor neurons (photographs A and B). This

10 difference in labelling intensity is probably due to

the fact that only a few motor neurons, very close to

the site of injection, were able to absorb the virus

intensely.

The anti-CGRP immunocytochemistry coupled to the

15 β-galactosidase revealing made it possible to

demonstrate, by a double staining, that practically all

the CGRP-positive cellular bodies (i.e. motor neurons)

were infected by the virus (photograph C).

20 <u>Effect on the immune/inflammatory response after</u>

<u>intramuscular injection of adenovirus:</u>

Immunity:

Epidemiological studies have suggested that as

much as 60% of the population over the age of 30 could

25 have antibodies against adenovirus type 5 (Matsuse et

al., Immunohistochernical and in situ hybridisation

detection of adenovirus early region 1A (E 1A) gene in

the microglia of human brain tissue. J. Clin. Pathol.,
47, 275-277, 1994). The existence of such a preimmunity
in humans could limit the efficiency of recombinant
adenovirus injection. In order to test this

5   possibility, mice were preinjected subcutaneously with
low doses of a defective Ad-RSVßgal (10^6pfu) in order to
preimmunize the animals. Three weeks after the
preimmunizing injection, Ad-RSVßgal ($2.5 \ 10^8$ pfu) was
administered intramuscularly. One week after

10   intramuscular injection, ßgal expression in the muscle
was compared in naive animals and preinjected animals.
Experiments were done using transgenic animals tolerant
for ßgal, in order to avoid any immune response against
the transgene. Experiments were done using either Ad

15   1.0 or 3.0 (dll007) vectors. ßgal expression in muscle
was quantified by luminometry analysis. The percentage
of enzymatic activity observed in preinjected animals
as compared to non preinjected animals is shown in
Figure 6.

20

Conclusion

      The data suggest that preinjection affects only in
a moderate fashion the efficiency of intramuscular
adenoviral gene transfer. Three weeks after

25   intramuscular injection, levels of transgene expression
in preinjected animals were 90% that observed in
animals that were not preinjected.

Inflammatory Response:

In order to compare the inflammation produced by

5 Ad 1.0 and Ad 3.0, mouse gastrocnemius muscle was

injected with 10^9 tdu of Ad 1.0 E3+ or Ad 3.0 (dll014)

encoding ß-galactosidase. Injections were performed

using transgenic animals tolerant for ßgal, in order to

avoid any inflammatory response against the transgene.

10 Two and seven days after injection, no inflammation was

vizualized in PBS injected muscle. In contrast every

Ad-injected muscle displayed a variable number of

inflammatory foci, with comparable intensity for Ad 1.0

E3+=Ad 3.0 (n=2 per condition). ßgal expression was

15 detected in myofibres, and also in endo- and epimysium.

The number of labeled muscle nuclei was higher with Ad

1.0 E3+ than with Ad 3.0.

Conclusion

These short term experiments do not demonstrate

20 an improvement of either inflammatory response or

number of labeled cells after i.m. injection of Ad 3.0

as compared to Ad 1.0. However it remains to be

investigated whether Ad 3.0 would confer an improved

stability of transgene expression on the long term.

25

Example 2.   Examples of the Present Invention in

Animals Recognized as Models of Amyotrophic Lateral

45

Sclerosis

Animal models

pmn mice: The progressive motor neuropathy (pmn)

5 mutants appeared spontaneously, and the disorder is

transmitted in an autosomal recessive fashion

(Schmalbruch and Skovgaard-Jensen, Progressive motor

neuropathy (pmm), a new neurological mutant in the

mouse. Mouse Genome, 87, 113, 1990). The mutated gene

10 is unknown but has been localized on chromosome 6.

Homozygous pmn/pmn mice suffer from caudo-cranial motor

neuron degeneration and die 5-7 weeks after birth,

probably due to respiratory muscle denervation.

Detailed histopathological studies of phrenic nerve

15 indicate that the disease is due to a dying back

process with distal axon degeneration, and relative

preservation of proximal axons and cell bodies

(Schmalbruch et al., A new mouse mutant with

progressive motor neuropathy. J. Neuropathol. Exp.

20 Neurol., 50, 192-204, 1991). Electromyographical

analysis (Kennel et al., Neuromuscular function

impairment is not caused by motor neurone loss in FALS

mice: an electromyographical study. Neuroreport, 7,

1427-1431, 1996; Kennel et al., Electromyographical and

25 motor perforrnance studies in thepmn mouse model of

neurodegenerative disease. Neurobiology of Disease, 3,

137-147, 1996) shows that pmn mice present

characteristic features found in ALS patients such as a
pure neurogenic pattern, the presence of fibrillations,
decreased motor conduction velocity. In contrast, other
ALS features such as enlargement of motor unit action
5 potential are not observed in pmn mice.
SOD* mice: Transgenic mice expressing mutated forms of
Cu/Zn SOD responsible for familial forms of ALS have
been constructed. Animals overexpressing human SOD
carrying a substitution glycine to alanine at amino
10 acid position 93 (SOD*) display a progressive
motoneuronal degeneration leading to paralysis and
death at 4-6 months of age (Gurney et al., Motor neuron
degeneration in mice expressing a human Cu,Zn
superoxide dismutase mutation.
15 Science, 264, 1772-1775, 1994). First clinical signs
consist of fine limb tremor around 90 days of age,
followed by a reduction in stride length at 125 days
(Chiu et coll., 1995). Histological studies reveal the
appearance of vacuoles of mitochondrial origin around
20 day 37, and a loss of motoneurons from day 90 (Chiu et
al., Age-dependant penetrance of disease in a
transgenic model of familial amyotrophic lateral
sclerosis. Mol. Cell. Neurosci., 6, 349-362, 1995).
Compensatory collateral reinervation can be observed at
25 the level of neuromuscular junctions (Chiu et al.,
1995). SOD* mice display all electrophysiological
Lambert 's criterias which characterize ALS (Kennel et

al., 1996). However the poor therapeutical effect of
riluzole on SOD* mice suggests that they could
represent the most severe forms of the disease.

5 **AdNT-3 Construct:**

The mouse NT-3 gene is isolated as a 1045 bp PCR-
fragment encompassing the complete NT-3 preprosequence
and cloned into the EcoRV site of a shuttle plasmid
containing the inverted terminal repeat (ITR) of the
10 adenoviral genome, encapsidation sequences and
adenoviral sequences necessary for subsequent
homologous recombination. Transcription of NT-3 is
controlled by the Rous Sarcoma Virus (RSV) LTR promoter
and terminated by poly A signals present in the NT-3
15 gene and in the 3' adjacent adenoviral pIX gene. The
replication-deficient adenoviral vector AdNT-3 is
deleted in regions E1 and E3, and is obtained by *in
vivo* homologous recombination of the linearized pAdNT-3
plasmid with the ClaI-restricted genome of AdRSVßgal
20 (Stratford-Perricaudet et al., Widespread long term
gene transfer to mouse skeletal muscles and heart. J.
Clin. Invest. 90, 626-630, 1992).

Example 2A: Administration of adenovirus encoding NT3
25 into muscle of *pmn* mice:

pmn-mice were injected with an adenoviral vector
encoding murine NT-3 under the control of an RSV

promoter, such as described above, at age 3-5 days. The
adenoviral dose (10^9 pfu in 100µl per animal) was
unilaterally injected into three muscle groups, the
gastrocnemius, the triceps brachii muscle and the long
5   muscles of the thoracic trunk.

Transgene expression after injection procedure was
first tested using an adenoviral vector (Adluc) coding
for the firefly luciferase gene. At day 25, more than
99% of luciferase activity was found in the injected
10   muscle groups with only marginal activity detected in
liver (0.4%), heart (0.1%) and lung (0.1%) and no
activity found in diaphragm and spleen.

After administration of the Ad-NT3 vector,
adenoviral NT-3 transcripts were detected in the
15   injected gastrocemius muscles from day 15 to day 35
which demonstrates expression for more than four weeks
after NT-3 gene transfer. No adenoviral NT-3
transcripts were detected in various other tissues with
the exception of spinal cord.

20   At day 25, AdNT-3 injected gastrocnemius muscles
deomonstrated a more than 100 fold-increase in NT-3
immunoreactivity (300ng/g wet weight in Ad-NT-3
injected muscles vs 2.4 ng/g in uninjected animals). At
the same time, NT-3 immunoreactivity was detected in
25   the sera of AdNT-3 treated pmn-mice and exceeded by
about 11 ng/ml the baseline in non-injected mice.

Ad-NT3 treatment improves the life span of *pmn* mice:

All non-treated *pmn*-mice died before day 57 and
their mean survival was 40.4±2.4 days (n=14) (Figure
4). The control vector Ad-lacZ, administered by
5   intramuscular injection, did not modify the survival of
the *pmn*-mice (40.0±2 .5 days, n=12). In contrast, *pmn*-
mice treated with the AdNT3 vector survived up to 89
days. Their mean lifespan was significantly improved to
61.3±2.5 days (n=16, p<0.001) which represents a 50%
10 increase in mean lifespan. Some *pmn*-mice treated with a
fivefold higher adenoviral dose (5×10^9 pfu) did not show
a further improvement of lifespan (49/57/61 days, mean
56 days).

15 Ad-NT3 improves neuromuscular function:
In order to precisely characterize the disease
progression in AdNT-3 treated *pmn*-mice an
electromyographical (EMG) study was performed, using
three EMG-parameters: i) spontaneous denervation
20 activity (fibrillations) in gastrocnemius muscle ii)
amplitude of the evoked response (compound muscle
action potential, CMAP) elicited by a supramaximal
electrical stimulus applied to the sciatic nerves
(recorded in gastrocnemius muscle), and iii)
25 spontaneous electrical activity recorded from the
diaphragm.

i) Fibrillations mark the onset of the functional

denervating process; they occured before day 17 both in

AdNT-3 treated pmn-mice and in non-treated pmn-mice.

ii) The amplitude of the CMAP of the gastrocnemius

muscle increased with age and reached in normal mice a

5   plateau of 80-90 mV at age 45 days. In non-treated

pmn-mice a maximum of 20-30 mV corresponding to 40% of

normal is reached around day 20 with no further

increase (Figure 5) presumably due to ongoing

denervation. AdNT-3 treatment induced a significant

10 recovery of the CMAP amplitude in pmn-mice: at 3 weeks

it represented 70% of normal mice. The amplitudes of

the CMAP in pmn mice did not differ between right and

left legs of unilaterally injected mice.

iii) Because denervation of the diaphragm presumably

15 determines the course of the disease in *pmn* mice, the

effect of AdNT-3 treatment on the electrical activity

of this muscle at day 35 was investigated. The

inspiratory phase was characterized by a burst of

activity whereas the expiratory phase was characterized

20 by electrical silence. The number of positive-negative

deflections ("turns") within the impulse pattern of the

burst is a measure for the number of motor unit

discharges. The number of "turns" per inspiratory burst

was reduced by 65% in non-treated *pmn*-mice as compared

25 to healthy littermates (n=4 in each group); the maximal

amplitude of the bursts was the same in both groups. In

pmn-mice treated with AdNT-3 the number of "turns" per

burst was not significantly enhanced, but the maximal
amplitude increased considerably as compared to
non-treated *pmn*-mice (Table 1).

5   Table 1

	Control (n=4)	ʾUntreated *pmn* (n=4)	NT3 treated *pmn* (n=4)
Mean duration of a burst of inspiration (ms)	138.8 ± 4.4	77.2 ± 7.7 ($P < 0.003$)[a]	99.1 ± 14.1 ($P < 0.037$)[b] ($P < 0.22$)[c]
Mean number of turns per burst	119.4 ± 6.0	40.7 ± 8.5 ($P < 0.001$)[a]	46.9 ± 5.5 ($P < 0.001$)[b] ($P < 0.57$)[c]
Mean of maximal amplitude (μV)	626 ± 95	695 ± 78 ($P < 0.61$)[a]	1062 ± 70 ($P < 0.011$)[b] ($P < 0.018$)[c]

[a]: Control *vs* untreated *pmn*
[b]: Control *vs* NT3-treated *pmn*
10   [c]: Untreated *pmn* *vs* NT3-*pmn*

It was 626+95mV and 695+78 mV in healthy litternnates
and non-treated pmn-mice, respectively, but was
1062+70mV in AdNT-3-treated pmn-mice (p<0.02). The
15 increase of the spike amplitudè within the respiratory
bursts suggests that AdNT-3 causes an increase of the
size of the motor units in *pmn*-mice, which evidences an
induction of peripheral or collateral sprouting of
motor axon endings. The conclusion from
20 electromyographical analysis was confirmed by
histological analysis of endplate innervation in
treated and control pmn animals.

Ad-NT3 protects against axonal degeneration

Prolonged lifespan and improved neuromuscular

5 function of AdNT3 treated *pmn* mice were reflected by an

increased number of phrenic nerve fibres, which

innervates the diaphragm. Light micrographs at day 25

showed that the number of axons in non-treated *pmn*-mice

and AdlacZ treated *pmn*-mice were reduced to 122±13

10 (n=6) and 120±11 (n=8), respectively, as compared to

263±8 myelinated fibres in normal littermates (n=4).

The number of myelinated fibres in the phrenic nerve of

AdNT3 injected *pmn* mice was significantly higher

(164±15, n=8, P<0.05), which corresponds to a 30%

15 reduction in the loss of myelinated fibers. At day 35,

when several non-treated *pmn*-mice had already died, the

AdNT-3 treated *pmn*-mice still contained more myelinated

fibres (130±7, n=8) than non-treated (118±4, n= 10) or

AdlacZ-injected *pmn*-mice (115±4, n=8), albeit this

20 difference was less pronounced than ten days earlier.

Potentiated effects of AdNT-3 and AdCNTF cotreatment

The effect of NT-3 is enhanced by CNTF, a molecule

known for its neuroprotective action in the *pmn*-model

25 (Sendtner et al., Nature 373, 344-346 (1995); Sagot et

al., Eur J. Neurosci. 7, 1313-1322 (1995). Mice were

treated with a combination of AdNT-3 and AdCNTF, an

adenoviral vector coding for a biologically active and

secretable form of CNTF. The mean survival of

cotreated *pmn*-mice (66±7.6 days, n=11, Fig. 8) tended

to be higher than for each treatment alone (AdNT-3:

5 61.3±2.5 days, n=16; AdCNTF: 52.9±4.4 days, n=13)

although this difference remains below statistical

significance. All *pmn*-mice however that survived for

more than 3 months (maximum 105 days) belonged to the

AdNT-3 + AdCNTF group.

10   At 25 days of age, animals cotreated with AdNT-3

and AdCNTF had 192±11 phrenic nerve fibers (n=8) while

animals treated with AdNT-3 or AdCNTF alone had 164±15

and 167±21 (n=4) fibers, respectively (Figs. 9 and 10).

At day 35, the number in the AdNT-3+CNTF group had

15 decreased to 157±10 (n=10), which is significantly

higher than in the AdNT-3 group (130±7, p<0.05) and in

the control groups (non-treated: 118±4, n=10; AdlacZ-

injected: 115±4, n=8, p<0.01 respectively). Hence, at

days 25 and 35, the loss of myelinated fibers was 30%

20 and 20% smaller in animals that had received AdCNTF in

addition to AdNT-3 than in animals that had received

AdNT-3 only.

Treatment with AdNT-3 and AdCNTF induces muscle

25 reinnervation

The electrophysiological findings are suggestive

of reinnervation phenomena in AdNT-3 treated *pmn*-mice.

The pattern of terminal innervation in superficial
gluteus muscles using an acetylcholinesterase-silver
method (Namba et al., Am. J. Clin. Pathol. 47, 773-783
(1967); Gurney et al., J. Neurosci. 12, 3241-3247

5   (1992)) was, therefore, examined. In normal mice, a
single terminal axon usually supplied one endplate
(Fig. 11 a and b) and only few axons branched at their
last Ranvier node and supplied two endplates. In
untreated *pmn*-mice the number of axons in intramuscular

10 nerve branches was greatly reduced, only few bundles of
terminal axons emerged from the nerves, and numerous
endplates lacked axons (Fig. 11c). Endplates still
connected to an axon showed the same one-to-one
innervation pattern as in normal mice: both the

15 terminal axon and its terminal branches had blunt
dilatations, and the terminal branches were rarified
(Fig. 11d). Argentophilic granules marked the course
of degenerated axons and were also within the nerve
branches. In some places fine sprouts emerged from the

20 end of broken axons and also from Ranvier nodes, but
these sprouts were thin (presumably non-myelinated) and
usually did not reach an endplate. The sensory
innervation of muscle spindles was preserved.

Figure 11e shows the pattern in one out of 3 AdNT-

25 3 treated *pmn*-mice. Bundles of terminal axons emerging
from the branches were more prominent and the axons
branched at successive Ranvier nodes and each supplied

several endplates.   After AdNT-3+AdCNTF treatment,

analyzed in 4 *pmn*-mice, the endplate zone of the muscle

was filled with criss-crossing axons which supplied

endplates arranged in a grape-like fashion.   In these

5   mice, endplates devoid of an axon were virtually

absent. Axons approaching endplates were short and most

emerged from Ranvier nodes but also often, from other

endplates (terminal sprouts, Fig. 11 f).   It was not

unusual to see a terminal sprout supplying an endplate

10   which then again had a terminal sprout supplying a

second endplate.

Conclusion of the pmn study

15   Intramuscular injection of an Ad-NT-3 provides a

50% increase in life span to *pmn* mice. The

physiological mechanisms likely to be responsible for

the effect of NT-3 on life span has been determined:

protection of axons from degeneration, and stimulation

20   of collateral sprouting at the level of terminal

endings.

The effects of combined AdNT-3 and AdCNTF

cotreatment on axonal survival and maximum lifespan of

pmn-mice were more pronounced than those of AdNT-3 or

25   AdCNTF alone.   This suggests that, in *pmn*-mice, these

two factors might act on distinct motor neuron

subpopulations, be important at different stages of

motor neuron degeneration or even influence
differentially non-neuronal cells like muscle cells
(Helgren et al., Cell 76, 493-504 (1994)) or Schwann-
cells. These findings also stress the therapeutic

5   potential of coadministering neurotrophic factors
belonging to different families.

These results constitute the first demonstration
of the impact of _in vivo_ gene transfer on life span in
a genetic animal model of a disease. Furthermore, this

10   study provides a strong rationale for the use of
adenoviral mediated gene therapy for motoneuronal
diseases. Indeed, comparable results can not be
obtained by systemic injection of recombinant NT-3
protein. Adenoviral-mediated administration of NT-3

15   modifies the bioavailability profile of NT-3 as
compared to systemic protein administration. The
protein is produced both at the post synaptic ends of
neuromuscular junctions, in motor neuron cell bodies
and is continuously released in the circulation.

20

Example 2B: Administration of adenovirus encoding
neurotrophic factors into muscle of SOD* mice:
Animal supply: The mutant animals are supplied by
Transgenic Alliance. SOD* animals present a wide

25   variability in life span, up to 50 days differences in
the life span of mice having received the same
treatment at the same time. As a consequence, a large

57

number of animals is necessary to reach statistically significant results.

General Methods

5   Animals were injected with different adenoviral constructs (AdNT-3, AdCNTF, AdBDNF, AdGDNF, Ad-ßgal) and at different ages. The evoked motor response in the gastrocnemius muscle after supramaximal stimulation of the sciatic nerve was recorded in each animal every

10   ten days. A comparison with riluzole was also undertaken.

Effect of intramuscular injection of Ad-NT3

Intramuscular injections were performed in newborn

15   and adult animals with an adenoviral vector encoding murine NT-3 under the control of an RSV promoter. Animals were injected either at 4-5 weeks of age, just before the appearance of the first electromyographical deficits, or at 13 weeks of age at the appearance of

20   the first clinical symptoms (Table 2).

Table 2

25

Age of injection	Ad-NT3	Ad-ßgal	animals not injected
newborn	163.9 ± 5.34 (n=12) P ^{2} 0.049 vs uninjected P^2 0.003 vs Ad-ßgal	145.17 ± 2.45 (n=10)	

58

4-5 weeks	161.26 ± 2.86 (n=15) P^2 0.045 *vs* uninjected P^2 0.048 *vs* Ad-ßgal	152.16 ± 3.32 (n=18)	152.04 ± 3.02 (n=24)
13 weeks	166.9 ± 4.17 (n=10) P^2 0.009 *vs* uninjected	N.D.	

N.D. not determined

The data show that intramuscular injection of

Ad-NT3 in SOD* mice produces an increase in life span

5   of 9-14 days as compared to uninjected controls. The

observed differences are statistically significant.

Interestingly, an increase in life span is observed

when animals are injected at 13 weeks, i.e. at the time

of appearance of the first symptoms.

10   In another experiment an adenovirus encoding

β-Gal or NT3 was injected to 3-4 weeks old $FALS_{G93A}$

mice, a strain of transgenic mice expressing a

mutated form of human SOD (93Gly -> Ala) and

displaying a progressive motoneuronal degeneration,

15   leading to paralysia of the limbs and death within

4 to 6 months (Gurney et al., 1994).

A total of 5.10^8 to 10^9 pfu was injected per

animal, distributed by quarter in each gastrocnemian

and biceps muscle. Lethality was determined. Untreated

20   animals lived 142.4+/-4.5 days, which is not

statistically different from the Ad-B-Gal injected

animals (147.8+/-3.0). However, Ad-NT3 injected animals

lived for 162.86+/-5.75 days. 6 out of the 7 ad-NT3-
treated animals lived even longer than the oldest non-
treated animals. 5 out the same group lived longer than
the oldest ad-G-Gal treated animals. These results

5   demonstrate than im injection of a neurotrophin-
expressing adenovirus can slow down disease progression
in a murine model of ALS.

The evoked motor response in the gastrocnemius
muscle after sciatic nerve stimulation was examined at

10 different ages. A very large variability was observed
for this parameter. A regular decrease was observed
along the course of the disease in each animal (Kennel
et al., 1996), but the rate and the extent of this
decrease could not be correlated with life span. No

15 significant modification of this parameter upon Ad-NT3
treatment was observed.

No significant effect on life span in SOD* mice
was observed when other recombinant adenoviruses were
injected intramuscularly, namely: Ad-GDNF, Ad-CNTF,

20 Ad-BDNF injected at birth; Ad-GDNF injected at 5 or 13
weeks; combination of Ad-GDNF+Ad-BDNF or
Ad-GDNF+Ad-CNTF injected at birth; or AdGDNF+Ad-CNTF
injected at 5 weeks.

25 Effect of Riluzole:

Riluzole is an FDA approved therapy for ALS.
Riluzole has also been shown to delay median time to

death in a transgenic mouse model of ALS. These mice

express human superoxide dismutase bearing one of the

mutations found in one of the familial forms of human

ALS. Riluzole is a member of the benzothiazole class.

5   Chemically, riluzole is 2-amino-6-(trifluoromethoxy)-

benzothiazole.

The effect of riluzole on SOD* mice was tested in

three different protocols. *p.o.* administration of 4 and

8 mg/day/kg to SOD* mice from the 5th week of age,

10 produced no effect on life span.

Riluzole was delivered in the drinking water of

SOD* mice at a concentration of 100µg/ml, a condition

reported to produce a 13 days increase in human patient

life span (Gurney et al., Pathogenic mechanisms in

15 familial amyotrophic lateral sclerosis due to mutation

of Cu,Zn superoxyde dismutase. Path Biol., 44, 51-56,

1996). This corresponds to an approximate dose of

20-25mg/kg/day, at which no effect on life span of the

SOD* mice was observed (treated animals 151.8+6.26,

20 n=11).

Conclusion:

Riluzole at concentrations ranging from 2.5 to 16

times those effective in human clinic

25 (100mg/patient/day, i.e. approx 1.5mg/kg/day)produced

no effect on SOD* mice life span. This suggests that

SOD* mice represents a severe form of human ALS. This

finding is reinforced by the fact that SOD* mice
represent a model for familial ALS, which is thought to
have an earlier onset and a poorer prognosis than
sporadic forms (Rowland, Amyotrophic lateral sclerosis:
5 Human challenge for neuroscience. Proc. Natl. Acad.
Sci. USA, 92, 1251-1253, 1995; Gurney et al., 1996).

The data show that Ad-NT3 injected intramuscularly
in SOD* mice produces increase in life span in the
range of 9-14 days as compared to controls. This
10 increase was significant. Three interesting features
must be noted:
a) Increase in life span was observed when injection
was performed in adult mice displaying the first
clinical signs of the disease.
15 b) No therapeutic or life span increase was observed
after the injection of NT3 protein into SOD* mice.
Therefore, as for *pmn* mice, the results demonstrate the
advantages of the delivery of therapeutic proteins
through gene transfer.
20 c) No increase of SOD* mice life span could be observed
in our hands with riluzole. This suggests that any
treatment producing an increase in life span in SOD*
mice can be predicted to produce a better effect than
riluzole in human patients.
25   Therapeutic effects may also be obtained by using
intrathecal administration (see WO94/08026), or with
viruses expressing other trophic factors.

Example 3: Intramuscular administration of naked DNA

       The data using *pmn* or SOD* mice show that

intramuscular injection of recombinant adenovirus

5   expressing a trophic factor can produce therapeutic

effect in these animals. The observed effects may be

linked to retrograde infection of motor neurons and to

trophic factor expression in the spinal cord.

Alternatively, the trophic factor could exert its

10 activity after being released in the circulation from

the infected muscle. In this case, any efficient method

for neurotrophic gene transfer in muscle could produce

beneficial effects similar to those observed with

adenoviral infection.

15   Gene transfer in skeletal muscle can be achieved

after by direct injection of naked DNA (Wolff et al.,

Direct gene transfer into mouse muscle in vivo.

Science, 247, 1465-1468, 1990). Several examples of

intramuscular transfer of genes encoding secreted

20 proteins have been published, where the level of

transgene expression was sufficient to produce a

physiological effect. A study of the potential

therapeutic potential of naked DNA transfer for   •

motoneuronal disorders has been undertaken.

25

Comparison of transgene expression levels after Ad and

naked DNA injection.

Mouse gastrocnemius muscle has been injected with
a recombinant adenovirus encoding luciferase (AdCMVLuc)
or a plasmid expressing the same transgene (pCMVLuc),
under the control of a CMV promoter. After injection of
5   10^9 pfu of AdCMVLuc, 100-300 pg of luciferase was
detected in the injected muscle. This value was
comparable to that observed after injection of pCMVLuc
(Figure 7, Panel a). However the variability within a
group was much higher when using naked DNA when
10   compared to the adenoviral vector. Indeed, a difference
of 2-3 logs in transgene expression levels where
typically observed when using naked DNA, while the
variations where within the order of 1 log when using
adenoviruses.

15

Analysis of parameters potentially influencing gene
transfer/expression efficiency:
Promotor RSV/CMV: RSV and CMV promoters provided
comparable transgene expression when used in newborn
20   mice. When injected in adult animals, the RSV promoter
lead to a level of luciferase expression that was more
than 10-fold lower than that observed with CMV 72 hours
after injection. However, 30 days after injection
similar levels were observed with the two constructs
25   (Figure 7, Panels c and d).

Age at injection: Injection in adult or newborn animals

produced comparable levels of transgene expression when
the sacrifice was performed 30 days after injection. In
contrast, when sacrifice was performed 72 hours after
injection, the levels of RSV driven luciferase

5   expression were more than 10 times higher in newborn
than adults, while the contrary was observed with a CMV
promoter (Figure 7).

Batch preparation: A ten-fold difference in luciferase
expression can be observed between two different

10   experimental batches of the same plasmid, prepared
using standard commercial kits (Figure 7). In contrast,
the levels of transgene expressed when using different
batches prepared by the production team were constant
(not shown). This stresses the need for controlled

15   conditions of plasmid preparations.

Muscle injected, sex, mode of injection: No major
difference depending on muscle injected (triceps vs
gastrocnemius), sex or mode of injection
(transcutaneous vs surgery) was observed.

20

Conclusion of the naked DNA experiments:
The results show that intramuscular injection *of* naked
DNA produces transgene expression levels comparable to
that observed after intramuscular injection of

25   adenovirus. However interanimal variability is much
higher with naked DNA than with adenoviruses. The data
also stress the need for controlled production *of*

plasmid to be injected.

All the references discussed herein are
incorporated by reference.

5   One skilled in the art will readily appreciate the
present invention is well adapted to carry out the
objects and obtain the ends and advantages mentioned,
as well as those inherent therein. The peptides,
polynucleotides, methods, procedures and techniques

10   described herein are presented as representative of the
preferred embodiments, and intended to be exemplary and
not intended as limitations on the scope of the present
invention. Changes therein and other uses will occur
to those of skill in the art which are encompassed

15   within the spirit of the invention or defined by the
scope of the appended claims.

CLAIMS

1.   A method of delivering one or more nucleic acids to mammalian motor neurons comprising administering said nucleic acids to muscle tissue, wherein said

5   nucleic acids are delivered to said motor neurons.

2.   A method of producing protein in mammalian motor neurons comprising administering one or more nucleic acids encoding said protein to muscle tissue, wherein said nucleic acids are delivered to said motor neurons

10 and expressed.

3.   A method according to claim 2, wherein said protein is produced at the post synaptic ends of neuromuscular junctions.

4.   A method according to claim 2, wherein the motor

15 neurons are afferent motor neurons and said nucleic acids are expressed in the spinal cord.

5.   A method of inducing peripheral or collateral sprouting of motor axon endings comprising administering one or more nucleic acids to muscle

20 tissue, wherein said nucleic acids induce peripheral or collateral sprouting of motor axon endings.

6.   A method according to claim 5, wherein the nucleic acids encode protein.

7.   A method of protecting against axonal degeneration

25 comprising administering one or more nucleic acids to mammalian muscle tissue, wherein said nucleic acids are delivered to said motor neurons and protect against

axonal degeneration.

8.   A method of treating an impairment of the nervous
system comprising administering one or more nucleic
acids encoding one or more neuroactive substances to
5 mammalian muscle tissue, wherein said nucleic acids are
delivered to motor neurons.

9.   A method according to claim 8, wherein the
impairment is nerve damage.

10.   A method according to claim 8, wherein the
10 impairment is a neurodegenerative disease.

11.   A method according to claim 10, wherein the
disease is amyotrophic lateral sclerosis or spinal
muscular atrophy of infancy.

12.   A method of treating amyotrophic lateral sclerosis
15 comprising administering to muscle tissue of a mammal
suffering therefrom a replication defective adenovirus
comprising a gene encoding neurotrophin-3 and a
replication defective adenovirus comprising a gene
encoding CNTF.

20 13.   A method according to claim 1, wherein the nucleic
acids are inserted in a vector.

14.   A method according to claim 13, wherein the vector
is selected from the group consisting of an adenovirus,
retrovirus, herpes virus and adeno-associated virus.

25 15.   A method according to claim 14, wherein the vector
is a replication defective adenovirus.

16.   A method according to claim 15, wherein the

adenovirus lacks all or part of the E1 region, and all

or part of the E3 and/or E4 region of the adenovirus

genome.

17.   A method according to claim 15, wherein the

5 adenovirus is an adenovirus of human origin.

18.   A method according to claim 15, wherein the

adenovirus is an adenovirus of animal origin.

19.   A method according to claim 15, wherein the

nucleic acid is inserted into the genome of the

10 adenovirus within the E1, E3 or E4 region.

20.   A method according to claim 1, wherein the nucleic

acids encode neuroactive substances.

21.   A method according to claim 20, wherein the

nucleic acids encode a growth factor, neurotrophic

15 factor, cytokine, neurotransmitter synthesizing enzyme,

enzyme, or receptor.

22.   A method according to claim 20, wherein the

nucleic acids comprise signals enabling expression of

the neuroactive substance in motor neurons.

20 23.   A method according to claim 1, wherein the motor

neurons are cervical medullary motor neurons, and said

administering is into muscles of the upper limbs.

24.   A method according to claim 23, wherein said

muscles are biceps and/or triceps.

25 25.   A method according to claim 1, wherein the motor

neurons are thoracic medullary motor neurons, and said

administering is into muscles of the thorax.

26. A method according to claim 25, wherein said muscles are pectoral muscles.

27. A method according to claim 1, wherein the motor neurons are lumbar and/or sacral medullary motor

5 neurons, and said administering is into muscles of the lower limbs.

28. A method according to claim 27, wherein said muscles are gastrocnemial muscles.

29. A method according to claim 1, wherein said

10 administering comprises injection at several points of the same muscle.

30. A method of delivering one or more nucleic acids to mammalian motor neurons comprising administering said nucleic acids to muscle tissue proximate to the

15 site of a nerve linkage associated with a chosen medullary functional level, wherein said nucleic acids are delivered to said motor neurons.

31. A method according to any one of claims 1, 2, 5, 7, 8, and 30, wherein said one or more nucleic acids

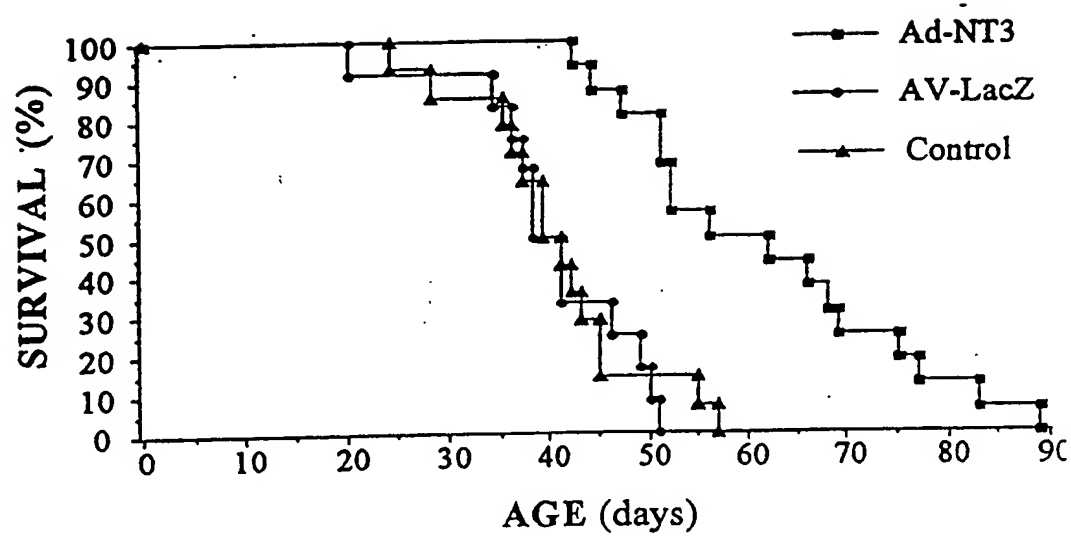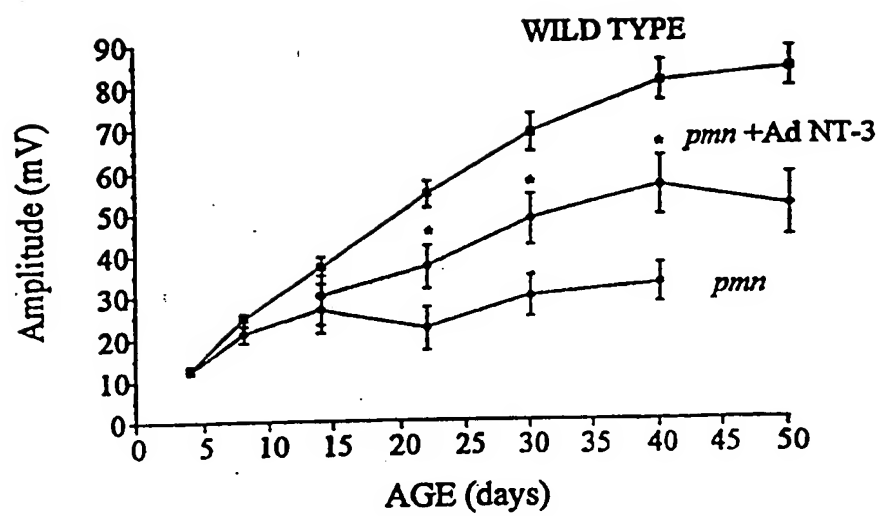20 encode neurotropin-3 and CNTF.
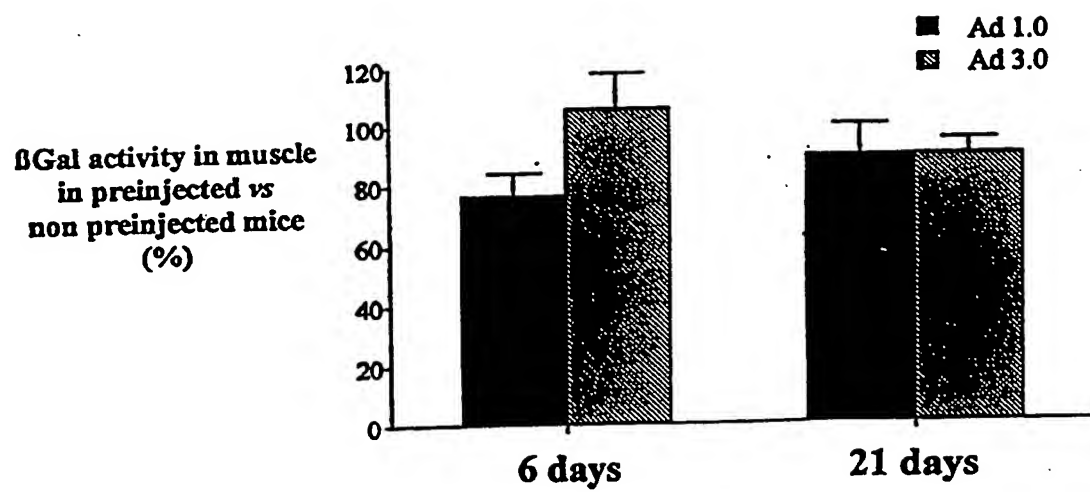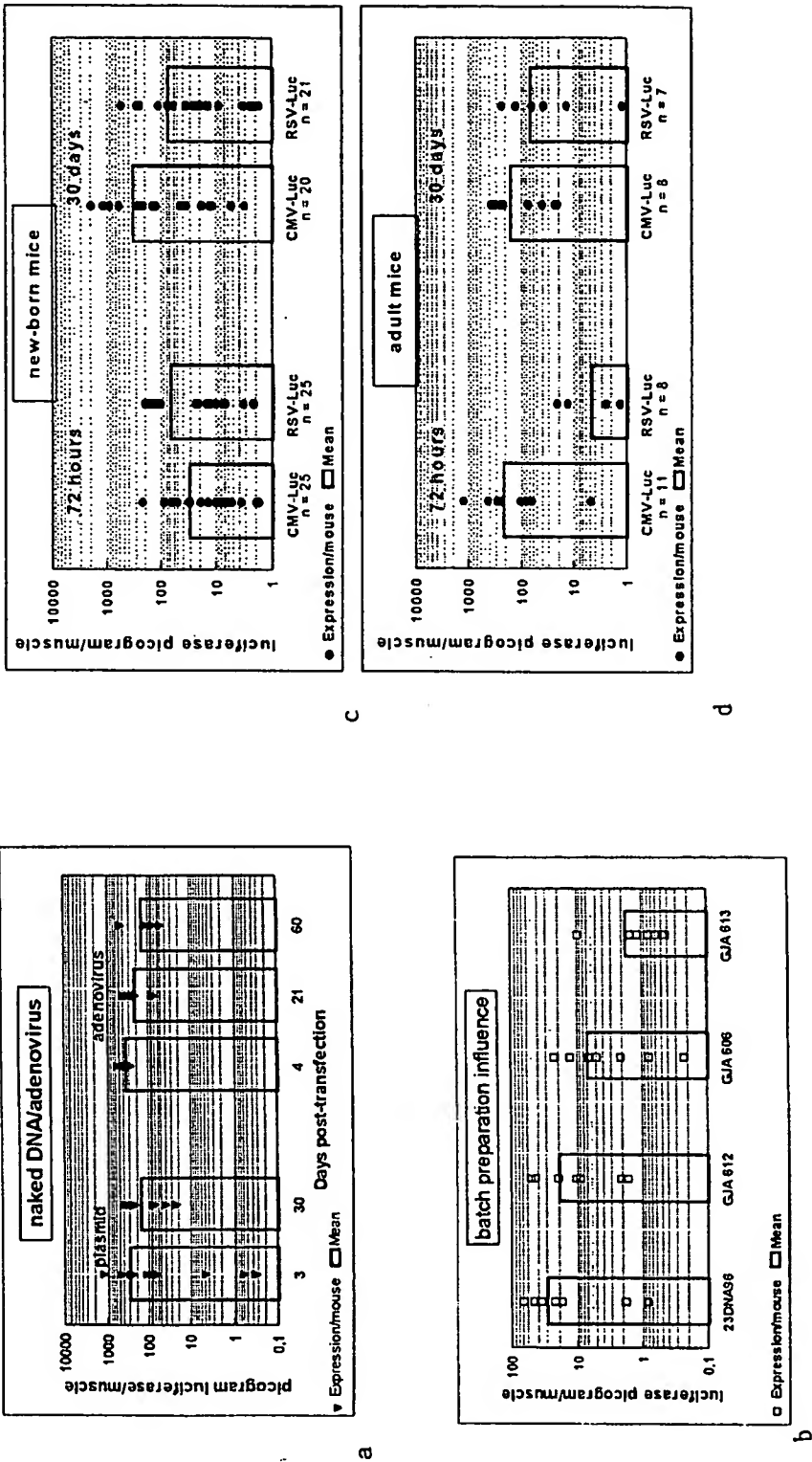
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   A61K48/00   C12N15/86

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   A61K   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 18740 A (RHONE POULENC RORER SA ;INST NAT SANTE RECH MED (FR); FINIELS FRAN) 20 June 1996 see claims 1-11	1-31
X	WO 96 27672 A (GLORIOSO JOSEPH C ;FINK DAVID J (US)) 12 September 1996 see page 14, line 10 - line 24; claims 12-20	1-31
P,X	WO 97 39629 A (CHOI LUNDBERG DEREK ;GENETIC THERAPY INC (US); BOHN MARTHA C (US);) 30 October 1997 see page 11, line 6 - line 28; claims 1-30	1-31

-/--

X Further documents are listed In the continuation of box C.   X Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 May 1998	02/06/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Seegert, K

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	KAHN A. ET AL: "Thérapie génique des maladies neurologiques" COMPTES RENDUS DES SEANCES DE LA SOCIETE DE BIOLOGIE ET DE SES FILIALES, vol. 190, no. 1, 1996, pages 9-11, XP002065157 see page 10, paragraph 1	1-31
X	GHADGE G D ET AL: "CNS GENE DELIVERY BY RETROGRADE TRANSPORT OF RECOMBINANT REPLICATION-DEFECTIVE ADENOVIRUSES" GENE THERAPY, vol. 2, no. 2, 1 March 1995, pages 132-137, XP000562924 "Discussion" see page 135 - page 136	1-31
P,X	HAASE G ET AL: "GENE THERAPY OF MURINE MOTOR NEURON DISEASE USING ADENOVIRAL VECTORS FOR NEUROTROPHIC FACTORS" NATURE MEDICINE, vol. 3, no. 4, April 1997, pages 429-436, XP002050329 see abstract	1-31

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9618740 A	20-06-96	FR 2727867 A	14-06-96
		AU 4350296 A	03-07-96
		CA 2208224 A	20-06-96
		CZ 9701806 A	17-09-97
		EP 0797677 A	01-10-97
		FI 972491 A	12-06-97
		HU 77258 A	02-03-98
		NO 972712 A	12-06-97
		SK 74597 A	10-12-97
WO 9627672 A	12-09-96	AU 4706996 A	23-09-96
WO 9739629 A	30-10-97	AU 2677797 A	12-11-97